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PRINCIPAL INVESTIGATOR: Jill E. Slansky

CONTRACTING ORGANIZATION: Regents of the University of Colorado
Aurora, CO 80045-2505

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Jill E. Slansky, John Kappler, Tullia Bruno, Daniel Munson, Taizo Nakano

E-Mail: Jill.Slansky@UCDenver.edu

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14. ABSTRACT

During the current funding period, we have started a collaborative effort between the laboratories of Peter Lee at City of Hope National Medical Center, Paul Spellman at Oregon Health Sciences University, and Jill Slansky at University of Colorado Anschutz Medical Campus. Our multi-team project is aimed at enhancing the function of pre-existing antitumor T cells from breast cancer patients. In the first year, we have focused on generating reagents and identifying conditions for experiments that will identify T cell antigens from patients with major subtypes of breast cancer. Towards this goal, we have achieved compliance with the Colorado and the Department of Defense Institutional Review Boards, accrued consented patients for optimization of study parameters, optimized conditions for isolation and confirmed function of T cells from breast cancer patients, finalized the protocol for amplification of T cell receptor RNA, sequenced the TCR β gene fragments from matched tumor infiltrating and blood T cells, and started to generate an HLA-peptide library. We are positioned to start validating antigens and developing antigenic peptides recognized by T cells from breast cancer patients.

15. SUBJECT TERMS
Breast cancer, tumor antigens, T cell receptor, cancer vaccine

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Table of Contents

INTRODUCTION:

One year into the Multi-Team Project ENHANCING THE BREADTH AND EFFICACY OF THERAPEUTIC VACCINES FOR BREAST CANCER, we have made clear progress at the University of Colorado site and as a team (the other teams are located at Oregon Health and Science University and the City of Hope). The major objective of this project is to develop novel strategies to enhance the protective effects of anti-tumor T cells *in vivo* in a patient-specific manner based on the hypothesis that partially protective anti-tumor T cells exist within in most breast cancer patients. The scope: this year we have generated many of the reagents and identified conditions necessary for future experiments. Specifically, we plan to identify the epitopes recognized by T cells in breast cancer patients and identify methods to improve the T cell responses to breast cancer.

The teams are working well together: we meet regularly on monthly conference call (organized by the OHSU group) and in person bi-annually (hosted to date by CoH and OHSU). In between formal meetings, the teams post results on a Google website (hosted by the OHSU group) and coordinate ongoing activities by phone and email. Most of the compliance issues are in place.

BODY:

The Tasks from the Statement of Work (revised 1-1-12) are in bold. Work done toward these Tasks is in regular font.

Task 1. Generate reagents and identify conditions for experiments to follow: months 1- 40, Lee, Slansky, and Spellman

1. The reagents that have been created/acquired and the experimental conditions that have been identified thus far will be discussed within the related tasks below.

A material transfer agreement among the three groups has been established and conditions for the distribution of cells (T cells, tumor cells, and tumor tissue) from CoH and from UC has been discussed and agreed upon. The process of establishing data use agreements (DUA) among the groups is underway. To date, the DUA at UC is in place so that we can receive a limited data set from CoH. The Institutional Review Board (IRB) protocols required to perform this project on human breast cancer samples is current.

Task 2. Enroll 100 patients with all major breast cancer subtypes from City of Hope and University of Colorado

2a. Identify subjects with the appropriate BC subtypes

2b. Obtain patient consent and procure samples

To date, 5 patient samples have been acquired to optimize our studies through Virginia Borges at the UC Anschutz Medical Campus. The collaborative has agreed for the Slansky lab to utilize these specimens to develop and validate protocols; i.e. these patients are not part of the 100 breast cancer patients representing the major subtypes that are proposed in the original application. We have obtained fresh tumor specimens, bloods, and tumor draining lymph nodes (TDLNs) to validate our protocols. The samples have been acquired under a UC IRB approved protocol with written informed consent obtained

Table 1. *Sample acquisition from University of Colorado Anschutz Medical Campus.*

Patient #	Subtype	HLA type (Class I/II)
UC0197	Luminal B	HLA-02*01/DR13*01
UC0198	Luminal A	In progress
UC0200	Luminal A	HLA-02*01/DR01*01
UC0202	Luminal A	HLA-01*01/DR07*01
UC0205	Luminal A	In progress

from each patient. None of the patients were previously treated with adjuvant therapy i.e. radiation, chemotherapy. Within two hours of surgery, the specimens are obtained and further processed. The subtype and HLA type of the patients currently enrolled in the study is summarized in Table 1.

Task 3. Process patient samples (blood, TDLNs, tumor): months 1-38, Lee and Spellman
3a. HLA typing of PBMC
3b. EBV transformation to generate B-LCL
3c. Disrupt and digest tumor and TDLN samples into single cell suspensions
3d. Isolate T cells by FACS sorting
3e. Expand T cells ex vivo

3a. HLA typing of PBMC. We are collecting peripheral blood from patients at the time of patient consent (1-2 weeks prior to surgery). After Ficoll separation of the lymphocytes, we isolate genomic DNA from $1x10^6$ PBMCs and send it to the City of Hope HLA typing lab. This facility is used by the Lee lab for typing of samples from CoH. The HLA results are summarized in Table 1. In addition, we acquired an antibody (BB7.2, Biolegend) that will allow for an immediate check of the patient HLA phenotype by flow cytometry. We will follow up this analysis by sending genomic DNA to the City of Hope HLA typing lab for the HLA genotype.

3b. EBV transformation to generate B-LCL. Performed by the Lee lab.

3c. Disrupt and digest tumor and TDLN samples into a single-cell suspension. Once the tumor and TDLN are collected, we process the tumor tissue according to Figure 1. TDLN are processed similarly, however, they do not require a digestion with the liberase enzyme. To date, we have obtained one TDLN (UC0200).

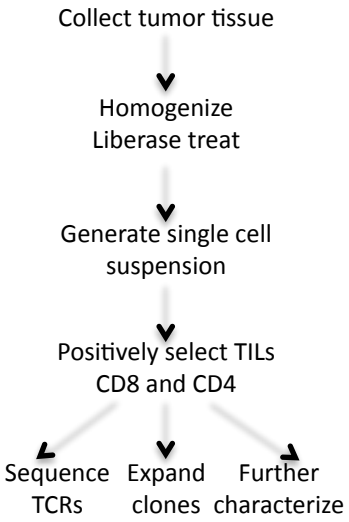

Figure 1. *Tissue processing and TIL selection.*

3d. Isolate T cells by FACsorting. CD8 and CD4 tumor infiltrating lymphocytes (TILs) are isolated using Dynal positive selection kits (Invitrogen). After positive selection, the overall purity of the samples is between 10-50% (Figure 2, top panel). The low purity is due to tumor cells being pulled through with the positive selection, not other lymphocyte contamination. In fact, analysis of the lymphocyte population showed the purity is over 90% for both the CD8 and CD4 TILs (Figure 2, bottom panel). These CD8 and CD4 TILs are then used for sequencing and in vitro functional studies.

Many studies show that detection of T cells in breast cancers is a positive indicator for patient survival (1). Thus, we predicted that T cells in breast cancer can be activated to kill the tumor, a central premise of our project. To show that CD8 and CD4 TILs can indeed be stimulated, we isolated T cells from tumor

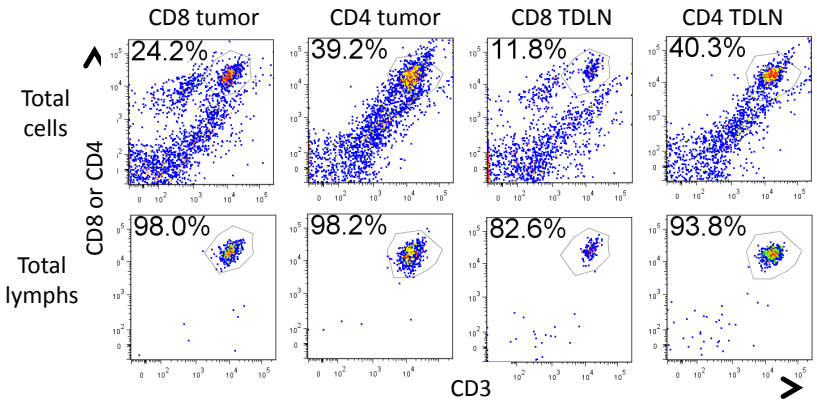

Figure 2. *Successful enrichment of TILs from breast cancer patients. There is significant patient-to-patient variation in the yield of T cells obtained from each sample. In addition, tumor development, tumor size, and sample size contribute to the final yield. We have obtained 5,000-150,000 T cells per sample (gate, lower panel). Sample UC0200.*

samples as above, and determined their function after stimulation with PMA/ionomycin or antibodies for CD3 and CD28. The first assay measured IFNγ production by CD8 and CD4 T cells from peripheral blood (PBMCs, positive control) and the tumor (TIL) (Figure 3A, B). Both produce IFNγ, which is a hallmark of immune function for T cells. A synopsis of this data from four patients is included (Figure 3B).

In addition to measuring IFNγ production, we also analyzed proliferation of CD8 TILs (Figure 3C). We found that CD8 TILs had increased proliferation when stimulated through the TCR for five days. Further, the population that proliferated also produced a robust amount of IFNγ. Although these functional assays do not show killing of tumor cells, this function is consistent with T cell killing. We are preparing to examine CD107 and CD154 on these cells in the near future (Task 4).

These results help to ascertain cell limitations and culture conditions for future functional studies.

3e. Expand T cells ex vivo. Lee lab is working on this.

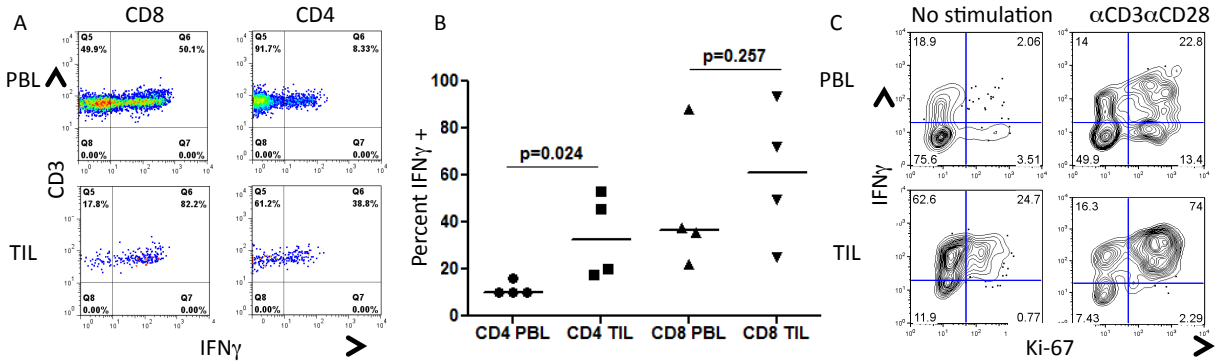

Figure 3. *Breast cancer TILs produce IFNγ and proliferate suggesting that they can be stimulated by a vaccine.* ***A, B****. CD8 and CD4 PBL and TILs were stimulated for six hours in vitro with PMA and ionomycin. This mitogen bypasses the TCR signal and nonspecifically stimulates the cell to produce cytokines. IFNγ production was determined by intracellular staining.* ***C.*** *CD8 PBMCs and TILs were stimulated with antibodies to CD3 and CD28. Ki-67 is a marker of proliferation. Many of the cells that produce IFNγ, also proliferate in response to stimulus. If the T cells were anergic and could not be stimulated, these results would be negative.*

Task 4. Identify and isolate anti-tumor T cells from TDLNs and tumor samples: months 1-40, Lee, Slansky, and Spellman

4a. Generate tumor lysates pooled from BC cell lines of each major subtype (luminal, HER2+, basal)

4b. Generate specific tumor antigen lysates from recombinant baculovirus-infected cells.

4c. Generate autologous tumor targets via B-LCL presenting tumor lysates

4d. Identify and isolate anti-tumor CD8 T cells via CD107 mobilization, and anti-tumor CD4 T cells via CD154 mobilization

TABLE 1. Known Breast Cancer Antigens	NCBI Reference	Sub-cellular Location	Expression in Tumor-Sub-Type Cell Lines
alpha-lactalbumin	NM_002289.2	Milk Protein	Low expression in culture, may be hormone induced
carcinoembryonic antigen (CEA)	M29540.1	Membrane glycoprotein	Luminal
HER-2/NEU	NM_004448.2	Membrane Tyrosine kinase	HER2+
Mucin 1 (MUC-1)	NM_002456.4	Membrane glycoprotein	Luminal and HER2+
NY-ESO-1	NM_172377.3	Transcription factor	Sporadic
NY-BR-1	AF269087.1	Membrane Protein	Subset of Luminal
Telomerase (TERT)	NM_198253.2	Nucleus	Low and sporadic

Table 2. *Known BC antigens (Table 1 from original application).*

4a. Generate tumor lysates pooled from BC cell lines of each major subtype. The Spellman lab is working on this.

4b. Generate specific tumor antigen lysates from recombinant baculovirus-infected insect cells. The purpose of these lysates is to screen the T cells against a panel of known BC antigens prior to screening libraries (Table 2). If the T cells react with these known antigens, the road to antigen and mimotope discovery will be greatly shortened. Toward this goal, we have started sub-cloning known breast cancer antigens into baculovirus for future protein expression and generation of lysates. In addition, we have started making cell lines that express and present these antigens. We obtained a cell line that expresses HLA-A*0201 (C1R:A2, from Vic Engelhard at University of Virginia). To date, we have stably transfected these cells with CEACAM5, NY-ESO-1, HER2, MUC-1, and hTERT.

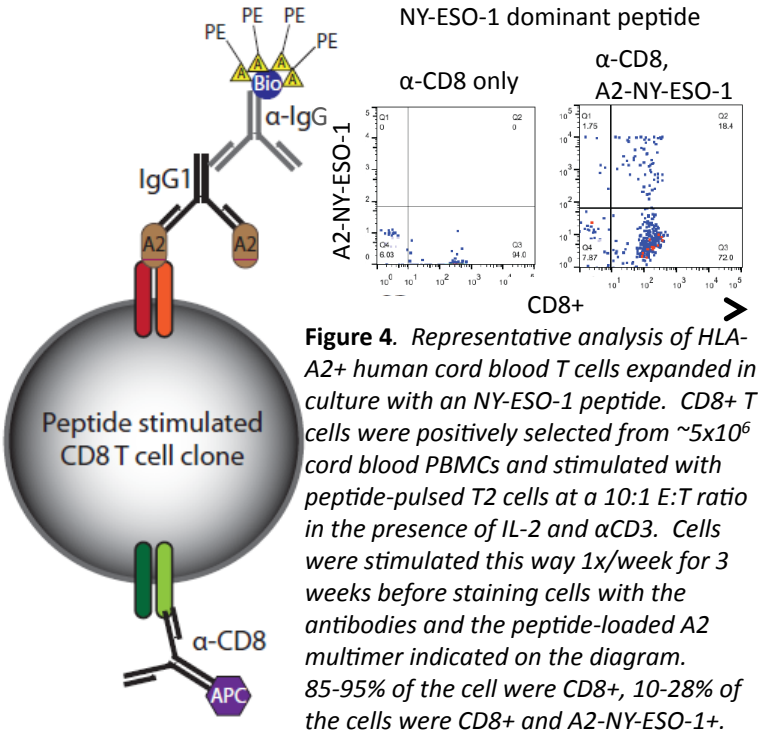

Figure 4. *Representative analysis of HLA-A2+ human cord blood T cells expanded in culture with an NY-ESO-1 peptide. CD8+ T cells were positively selected from ~5x10^6 cord blood PBMCs and stimulated with peptide-pulsed T2 cells at a 10:1 E:T ratio in the presence of IL-2 and αCD3. Cells were stimulated this way 1x/week for 3 weeks before staining cells with the antibodies and the peptide-loaded A2 multimer indicated on the diagram. 85-95% of the cell were CD8+, 10-28% of the cells were CD8+ and A2-NY-ESO-1+.*

Next, we must confirm that the specific HLA-A*0201-restricted tumor antigens are expressed by these cells. We are generating antigen-specific T cells by stimulating cord blood T cells in culture with known peptides of breast cancer antigens loaded onto T2 cells in the presence of IL-2 and CD3 antibody. Figure 4 shows how we are isolating the antigen-specific T cells. We will sub-clone the TCRs, insert them into hybridoma cell lines to make transfectomas that can be used to indicate antigen expression on the C1R:A2-tumor antigen cell lines. This methodology will be helpful when screening the peptide libraries as the total number of T cell clones may be limiting.

The generation transfectomas is detailed in Figure 5. Briefly, RNA is extracted from a T cell clone, a single T cell, or a complete TIL sample. A unique anchor is ligated to the 5' end of all transcripts and the RNA reverse transcribed into cDNA. PCR is

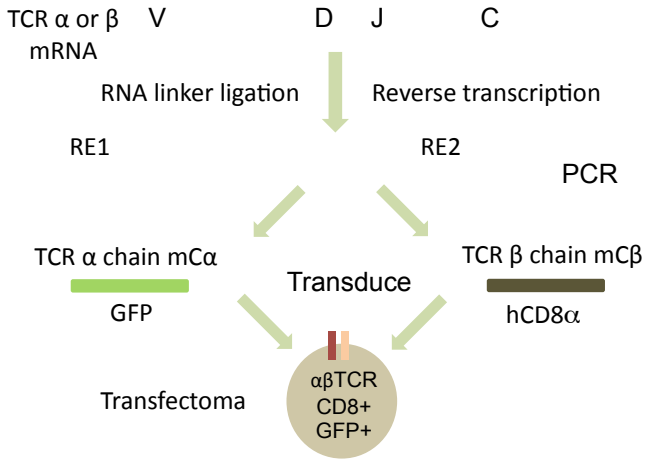

Figure 5: *Creation of a transfectoma using a specific αβTCR pair. TCR mRNA is isolated from T cells, linked to a known RNA sequence and reverse transcribed. PCR using primers specific to the constant region and to the known linker sequence creates a clonable TCR to put into a MSCV retroviral plasmid. The TCR α plasmid contains GFP under an IRES, and the TCRβ plasmid the human CD8α chain (with a mouse transmembrane domain) under control of an IRES. TCR+, GFP+, CD8α/β+ cells can be sorted by flow cytometry.*

performed with either an alpha or beta TCR reverse primer that binds to a conserved sequence in the constant region, and a forward primer specific to the linker. The reverse primer has a restriction site tail to aid in cloning. The PCR product is digested and cloned into plasmids used in transducing a T cell hybridoma that has little to no natural TCR expression, but expresses human CD8. The plasmids also contain GFP under an IRES used for cell sorting. TCR functionality can be assessed by binding to a potential target and measuring the release of IL-2 by ELISA.

Task 5. Generation and initial analysis of T cell clones: months 1-40, Lee, Slansky, and Spellman
5a. Expand isolated T cells as clones
5b. Confirm tumor reactivity and HLA restriction of each clone
5c. TCR sequencing of each clone
5d. Select 10 unique CD4 and 10 unique CD8 clones from each patient for further analysis

5. The purpose of generating and analyzing the T cell clones is to determine which T cells should be used for screening peptide libraries and ultimately identifying tumor epitopes and mimotopes. Since the number of different T cells that respond to a specific antigen can be large, we are analyzing the repertoire of T cells that infiltrate the tumor so that we choose "representative" T cells to screen the peptide libraries. We are analyzing the TIL repertoire using traditional and high throughput sequencing. We have made significant progress in developing standardized protocols to carry out this goal as discussed below. Both of these methods will suggest which T cell clones to obtain from the Lee lab and which should be used to generate reagents to screen peptide libraries.

After identifying overrepresented α and β TCR genes from representative BC TILs, we will make transfectomas to facilitate antigen and library screening. Our goal is to identify overrepresented families of VJ and V(D)J TCR recombinations (CDR3 regions) in the repertoire analysis that represent T cells that have been activated and have undergone clonal expansion. These activated T cells and their unique TCR will be utilized to screen for functional activity against known breast cancer antigens and identify novel tumor antigens/mimotopes as candidates for vaccine immunotherapy.

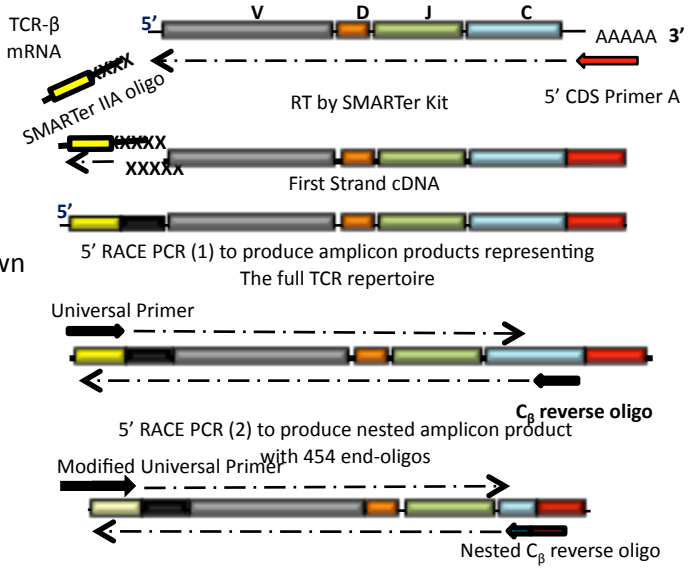

Figure 6. *TCR amplicon library protocol. cDNA is synthesized using the SMARTer kit. 5'RACE PCR is carried out in two sequential reactions. The initial reaction (PCR1) amplifies from a known sequence in the conserved region of the TCR template to the 5' end. The second nested reaction (PCR2) adds additional end sequence to both the 5' and 3' end to prepare the amplicon for sequencing.*

In the previous 12 months, we have developed a protocol to carry out 5' RACE high throughput sequencing to produce the TCR repertoire of both α and β TCR chains from BC TILs. Briefly, total RNA is extracted from the isolated T cell populations (Figures 1 and 2). cDNA is synthesized from total RNA using the SMARTer 5'RACE preparation kit, as described in Figure 6 (Clontech). SMART, Switching Mechanism at 5' End of RNA Template, is a modality that allows for efficient incorporation of known sequences at both ends of cDNA during first strand synthesis, without adaptor ligation. These known sequences are crucial to carry out downstream 5'RACE PCR. We chose to use this technology based on its efficiency of the single-step

SMART cDNA synthesis process. Rapid Amplification of cDNA Ends (RACE) is a technique used to obtain the full-length sequence of an RNA transcript found within a cell. RACE can provide the sequence of an RNA transcript from a small known sequence within the transcript all the way to the 5' end. For our current study, we will sequence from the conserved (C) region of TCR transcript to the 5' end to obtain the entire V(D)J region. The first round 5'RACE PCR produces sequences that represent the full TCR repertoire for either the α and β chain. These same PCR products can also be cloned into a TA vector for traditional sequencing. Nested PCR is carried out to purify the amplicon library and add both 5' and 3' end oligos required to carry out 454 Roche high throughput sequencing.

T cell populations from patient UC0197 were isolated at high purity and were selected for a sequencing validation study by cloning the TCR fragments into plasmids and analyzing by Sanger sequencing. Total RNA was isolated, cDNA was synthesized, and 5'RACE PCR was carried out following the protocol above (Figure 7). PCR1 produces an amplicon library averaging ~600 base pairs (bps). The amplicon product includes all bps from the 5' end through the full V(D)J TCR sequence. The secondary nested PCR2 product adds additional 5' and 3' end sequence required to carry out downstream high throughput sequencing, so the product also averages ~600 bps. The final TCR amplicon library undergoes a series of purification steps prior to any form of sequencing studies.

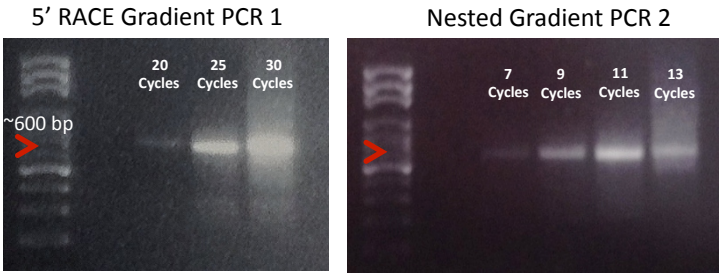

Figure 7. *Protocol validation on BC sample UC 0197. Gel electrophoresis of gradient PCR1 and gradient PCR2 are shown. We select a band in each gel in which the PCR product is in the linear phase of amplification and represents an amplicon library. We avoid bands that suggest over amplification of the TCR library, which may bias the resulting TCR repertoire sequencing results.*

We completed traditional sequencing of the CD8+ TCR β chains from TILs and PBMCs from patient UC0197. IMGT/V-QUEST (http://www.imgt.org/) was used to assign variable (V) and joining (J) TCR gene fragment to each sequence. Table 3 summarizes the preliminary results for patient UC0197, which have validated our protocols for cDNA production and 5'RACE amplification. Here we compare the TCR

	TILs		PBMCs		TILs		PBMCs	
V region gene	Frequency	#/46 total seqs	Frequency	#/108 total seqs	J gene pair	#/total V reads	J gene pair	#/total V reads
Homsap TRBV7-9*03 F	13.04%	6	2.78%	3	Homsap TRBJ2-2*01 F	6	Homsap TRBJ2-5*01 F Homsap TRBJ2-3*01 F	2 1
					Homsap TRBJ1-1*01 F	3		
Homsap TRBV28*01 F	10.87%	5	1.85%	2	Homsap TRBJ1-2*01 F	1	Homsap TRBJ2-7*01 F Homsap TRBJ1-1*01 F	1 1
					Homsap TRBJ2-2*01 F	1		
Homsap TRBV6-2*01 F,	10.87%	5	0.93%	1	Homsap TRBJ1-2*01 F	5	Homsap TRBJ1-2*01 F	1
or Homsap TRBV6-3*01 F					Homsap TRBJ2-2*01 F	1		
Homsap TRBV11-2*01 F	8.70%	4	-	-	Homsap TRBJ2-7*01 F	3	-	-
					Homsap TRBJ1-5*01 F	1		
Homsap TRBV18*01 F	6.52%	3	-	-	Homsap TRBJ2-5*01 F	3	-	-

Table 3. *Comparison of TCR sequences from TILs and PBMCs suggest an enrichment of a tumor-specific TCR in TILs. CD8+ T cells were isolated as in Figures 2 and 3, TCR sequences were amplified as in Figures 7 and 8, and subcloned into plasmids for sequencing. 46 TILs (red) and 108 PBMCs (black) β gene fragments were identified. The TCR VJβ sequences identified in the TILs differed from those in the PBMCs, consistent with tumor specificity.*

sequences from CD8+ TILs and PBMCs. Although the two repertoires share some gene fragments, there are over-represented V and J families in TILs that are not identified in PBMCs. The presence of over-represented VJβ sequences suggests the clonal expansion of T cells in response to antigens present in the tumor. Currently, we are undergoing TCR α chain sequence analyses.

Task 6. Generate MHC/peptide baculovirus libraries: months 1-40, Slansky
6a. HLA-A*0201
6b. HLA-DR*0401
6c. HLA allele most common in enrolled patients with basal tumors

Task 6a: Peptides libraries for HLA-A*0201 are currently being constructed and the library size determined. The peptide library sequence constraints are XL/MLXXXXXL/V, yielding a potential library size of 2.56×10^8 possible peptide combinations. See Figure 8 and (2) for cloning scheme for producing insect cells expressing single peptide-MHC combinations.

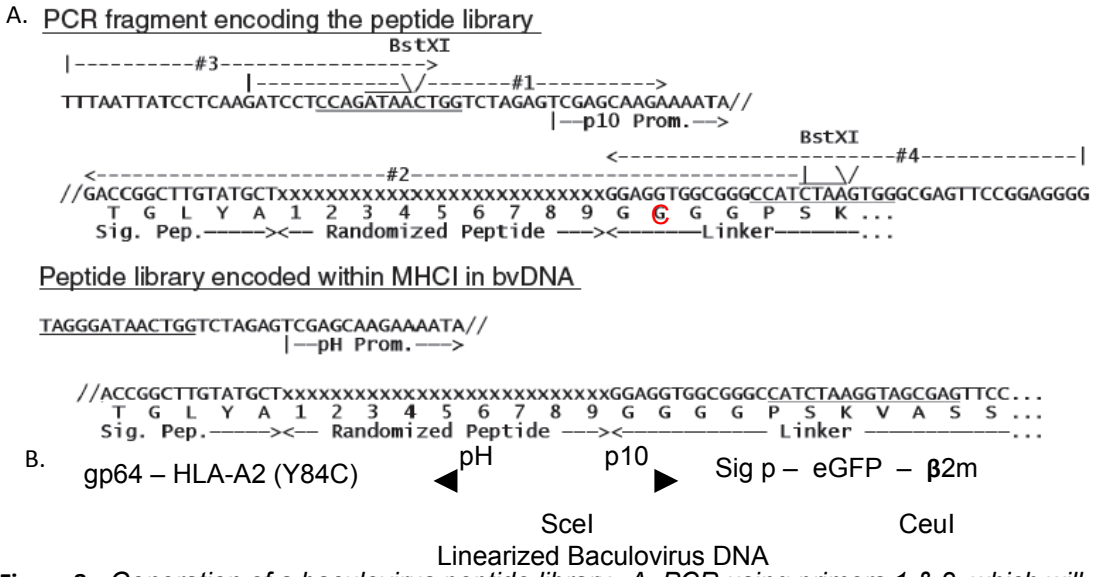

Figure 8. *Generation of a baculovirus peptide library. A. PCR using primers 1 & 2, which will generate a random peptide containing the motif detailed in the text creates a cleavable fragment which can be cloned into the SceI/CeuI sites on the baculovirus genome (B). The pool of circularized baculoviruses is then transfected into Sf9 insect cells and the library size determined by the lack of GFP staining and gain of MHC class I staining.*

Subsequent tasks have not yet been addressed.

KEY RESEARCH ACCOMPLISHMENTS:

- Achieved compliance with the Colorado and the Department of Defense Institutional Review Boards
- Accrued consented patients for optimization of study parameters
- Optimized conditions for isolation of T cells from PBMCs, tumor, TDLN
- Confirmed TIL function
- Finalized the protocol for 5'RACE TCR amplification
- Sequenced the TCR β gene fragments from matched TIL and PBMCs
- Started to generate an HLA-A*0201 peptide library

REPORTABLE OUTCOMES: None

CONCLUSION:

During the current funding period we, the Slansky and Kappler groups, have started a collaborative effort with Peter Lee at City of Hope and Paul Spellman at Oregon Health & Science University. Our multi-team project is aimed at enhancing the function of pre-existing antitumor T cells from breast cancer patients. This year, we focused on generating reagents and optimizing conditions for experiments that will identify T cell antigens from breast cancer patients. We were brought on to this team for our expertise with models of immunotherapy and T cell biology. We have made significant progress as shown in red in Figure 9. We have not encountered insurmountable hurdles during this period. We remain engaged preparing for the future tasks of identification of breast cancer–specific T cells and learning how to best activate these T cells. We have implemented three minor changes from the original application, which may expedite our progress.

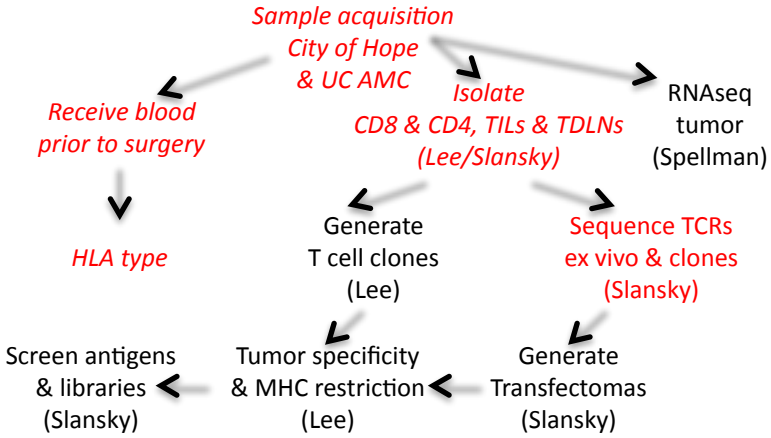

Figure 9. *Flow chart focusing on some of the details of the Slansky/ Kappler labs to identify breast cancer antigens. To date, the procedures in red have been executed for at least one sample.*

1. We are generating a panel of HLA-A*0201+ cell lines transduced with genes encoding known breast cancer antigens listed in Table 2. These cell lines, in addition to the recombinant baculovirus-infected insect cell lysates originally proposed, will facilitate screening the known antigens.

2. As a positive control for these cell lines, we are generating transfectomas, which are hybridoma cells transfected with specific T cell receptors (Figure 5). These cells secrete IL-2 upon binding of cognate antigen. With this technology in place, it will be straightforward to make transfectomas from the TCRs that are significantly represented in tumor-specific T cell repertoires and, if necessary, from clones that will not sufficiently expand for required applications.

3. Research performed in our labs and others (3) showed that significant skewing of the T cell repertoire can take place with T cells grown in culture in the presence of tumor or tumor antigen, likely because the effective antigen concentration is high (4) favoring “non-representative” clones to expand. T cells expanded with CD3 and CD28 antibodies, as we proposed in the original application and is performed in the Lee lab, were not included in our experiments. These skewing results have provided rationale to determine the sequence of the TCRs of the repertoire not just after expanding T cell clones, as we have proposed, but also ex vivo. We plan to execute these experiments using traditional sequencing of plasmids and high throughput sequencing (Figures 6, 7, Table 3); we have not yet optimized the inverse PCR analysis proposed in the original application. The sequencing results will also determine the similarity of the T cells in the tumor, TDLN, and the blood of breast cancer patients, necessary for efficient future epitope and mimotope discovery.

In conclusion, we are positioned to validate antigens and develop antigenic peptides recognized by T cells from breast cancer patients that will be valuable in the design of therapeutic vaccines for breast cancer.

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